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ORIGINAL ARTICLE

The differential roles of caspase family members in mediating PF4-induced breast cancer apoptosis

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Abstract

Introduction: This study focused on PF4 effects on caspase-3,-6, -7, -8 and -9 which regulate the apopotosis process in breast cancer. *Materials and Methods:* Breast tumours were induced in forty 21-day-old female Sprague Dawley rats (SDRs) using MNU until tumour size reached 14.5 mm (SD: 0.5 mm). The rats were then divided into two groups: Group 1 (control injected with 0.9% saline; n = 20), and Group 2 (platelet factor 4 (PF4); n = 20). PF4 was administered through focal intralesional injection at 20 μg/lesion dose. Following 5-day treatment, the SDRs were sacrificed. Subsequently, representative sections from the tumour were obtained for haematoxylin and eosin (H&E) staining. The expressions of caspase-3, -6, -7, -8 and -9 were evaluated using immunohistochemistry (IHC) staining. *Results:* The majority of breast tumour specimens were of aggressive types [$n_{control}$ = 13 (65%); n_{PF4} = 12 (60%)]. Invasive ductal carcinoma not otherwise specified (IDC-NOS) was the most commonly observed breast tumour histology for control and PF4 groups (n = 8 (40%) in respective groups). PF4-treated group exhibited significant differences in the caspase-3, -6 and -8 expression levels compared to the control group (all $p < 0.001$). There were no significant differences in caspase-7 ($p = 0.347$) and caspase-9 ($p = 0.373$) expression levels between both groups. *Conclusion:* This study found that PF4 acts via the caspase-mediated extrinsic apoptosis pathway without the involvement of the intrinsic pathway.

Keywords: PF4, breast cancer, caspase, apoptosis

INTRODUCTION

Apoptosis is critical to the metazoan development and homeostasis.[1] Apoptotic dysregulation leads to various human pathologies including cancer, autoimmune and neurodegenerative disorders. Apoptosis was first recognised in the year 1972 as an important regulator of cellular maintenance and turnover. Since then, research endeavours have led to the identification of hundreds of genes controlling the initiation, execution and regulation of apoptosis in several species.[2,3] Hence, these indicate that the apoptotic mechanism is evolutionarily conserved.[4]

Cysteine proteases (caspases) are the central components of apoptosis machinery. They cleave their substrates at aspartate residue and irreversibly induce cell death.[5] There are two distinct apoptosis pathways govern by caspases: the intrinsic (mitochondrial) and extrinsic (death receptor) pathway.[6] In the former, damage to the DNA caused by ultraviolet (UV) radiation and chemotherapeutic agents triggers the release of cytochrome c from the mitochondrial intermembrane space into the cytosol which then associates with apoptosis protease activating factor 1 (APAF-1) to form an apoptosome.[6] This structure will recruit and activate the initiator caspase-9 leading to caspase-3 and caspase-7 activation and subsequent apoptosis. In the latter, the binding of death receptor ligands (e.g.

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FasL and TNF-related apoptosis-inducing ligand (TRAIL)) to their respective death receptors (e.g. TNFR-1 and FAS/CD95) induces death receptor clustering and subsequent adaptor protein (e.g. Fas-associated death domain (FADD)) recruitment for the establishment of death-inducing signalling complex (DISC).[6] This chain of events will eventually cause the downstream activation of caspase-8 and subsequent executioner caspases; caspase-3, -6 and -7 that in turn leads to chromatin condensation, DNA fragmentation and other hallmarks of apoptosis.[6]

Many studies were conducted to identify factors which influence apoptosis pathways. Glutamic acid-leucine-arginine (ELR)-negative chemokine platelet factor 4 (PF4) has been found to induce the apoptosis of colon carcinoma and melanoma cells in xenograft mouse models.[9,11] PF4 was initially identified as an antiangiogenic agent.[7,8] It was shown to inhibit endothelial cell proliferation, migration and angiogenesis in both *in vitro* and *in vivo* models for various cancers.[8,9,10] We had also previously demonstrated that PF4 upregulated the pro-apoptotic Bax expression and downregulated anti-apoptotic survivin expression in an *in vivo* breast cancer model resulting in overt tumour shrinkage.[12] However, the effects of PF4 on each caspase and indirectly on the apoptosis pathways are still not entirely understood.

Due to the close interconnection between Bax and survivin and caspase family members in apoptosis modulation, we hypothesise that the increased Bax and reduced survivin activities will result in raised caspase family member expression levels. To verify this, the effects of PF4 on the caspase family members' expression levels were investigated.

MATERIALS AND METHODS

Animal preparation

Forty (n = 40) pre-pubertal female Sprague Dawley rats at the age of 21 days were obtained from the Animal Research & Services Centre of Science University of Malaysia (ARACS) and maintained at the Animal House Unit. The mean weight of the rats was 60 grams (standard deviation (SD): 4.4 grams). The rats were placed in 595 x 380 x 200 mm polycarbonate cages (1354G Eurostandard Type IV, Techniplast; Leicester, UK) with a ratio of 3 rats per cage. The rats were given standard rat maintenance diet (Altromin #1321®, Altromin International; Lage, Germany) and water liberally. The rats were acclimatised in 7 days to the following environmental conditions; temperature at 24°C (SD: 1.5°C), humidity at 55% (SD: 5%, range = 40% - 70%), lighting at 200 lux at bench level and a light dark cycle of 12:12 hours ratio with light switched on at 7.00 am and turned off at 7.00 pm daily. Wood-chip bedding (CHIPSI Classic®, JRS Group; Rosenberg, Germany) was changed every 3 days to ensure hygiene and prevent infection from occurring at the lesions. The rats were monitored until they were able to maintain upright posture and walking normally before the experimental procedures were implemented. The ethical clearance for using experimental animals was obtained from the USM's Animal Ethics Committee [reference no: PPSG/07 (A) /044/ (2010)]. The content and outline of this report follow the Animal Research; Reporting *In Vivo* Experiments (ARRIVE) guideline for animal research reporting and a filled-in ARRIVE checklist is provided at the end of this report[13].

Sample size calculation

No previous information on the SDs of each outcome was available from prior studies. Besides, to carry out a pilot study to determine the SDs, we require an additional 12 samples per intervention arms based on sample size rule of thumb for pilot study[14] which is against the principles of 3Rs (Replace, Refine and Reduce).[14] As a result, the sample size calculation based on power analysis as recommended by Cohen and co-workers could not be performed.[15, 16]

We thus resorted to the resource equation approach to determine the required sample size.[17] Mead and colleagues recommended 10 to 20 degrees of freedom (df) are sufficient to get a good estimate of error. Based on the formula for the error term in one-way ANOVA, the sample size formula is given by:

$$N_{group} = (df / k) + 1$$

If we set df = 20 and k = 2 groups, the sample size is therefore 11 per groups (N_{total} = 22). However, based on our experience, these will cause low precision and large uncertainties for each estimate if the sample size is too small., We hence deemed that 20 rats per groups were sufficient to obtain better estimates of the SDs and the effect size and this decision is also in agreement with the recommendations by Cox and Reid.[18] The more precise estimates will significantly aid in the planning and execution of future studies that extend our work here.

MNU & PF4 preparation and tumour induction

Prior to tumour induction, the crystallised form

of N-methyl-N-Nitrosourea (MNU, Sigma Aldrich®, St Louis, Missouri; Cat No: N4766) was dissolved at room temperature in a 0.2 ml of 0.9% normal saline that was acidified to pH 4 using 0.05% acetic acid by gentle heating and vigorous shaking to achieve a MNU concentration of 20 μg/mL. The MNU, dosed at 70 mg/kg, was then injected intraperitoneally into the rats on day 1 and 3 of the study.[19] The rats were weighed, palpated twice weekly, and visually-inspected daily for the onset of mammary tumour lesions for 30 days after MNU administration. The changes in tumour size were monitored and assessed by measuring the subcutaneous mass using Vernier caliper.

PF4 (Calbiochem, Darmstadt, Germany; Cat No: 521726) was prepared by dissolving 4 μL of 0.52 mg/mL PF4 stock solution in 96 μL of 0.9% saline to achieve a final PF4 concentration of 0.52 mg/mL based on Benny and co-workers' method.[20]

Experimental design

Malignant mammary gland lesions were monitored for 90 days post-MNU injection. Block randomisation was performed when allocating the rats into two groups after lesions reached a mean tumour diameter of 14.5 ± 0.5 mm. This was performed using a random number generator created by randomizeR package version 1.3 in R software by an independent third person.[21,22] The allocation sequence was then placed in sealed opaque envelopes to ensure allocation concealment and avoid selection bias.

To reduce the number of animals used, the block randomisation procedure was performed in 2 separate blocks, each having a size of 20. After the completion of experimental procedures in the randomised block 1, data was processed and analysed to assess whether the whole experimental effort would likely be futile. This is akin to the interim analysis (or futility analysis) in a clinical trial to evaluate whether the evidence of an effect is wholly lacking or absent. Since such trend was not found in our experiments, we proceeded with the second randomised block. The interventions were prepared and filled in colour-coded syringes by two independent individuals to ensure blinding. The study investigator who obtained the tumour samples and recorded the outcomes (Tengku Ahmad Damitri (TAD)) was also blinded to the intervention status.

Before injecting the allotted interventions, the rats were anesthetised intraperitoneally with a mixture of ketamine-HCl and xylazine dosed at 100 mg/kg and 10 mg/kg, respectively. Fresh physiological normal saline was then singly injected into each lesion on day 0 and 2 of the period of intervention in group 1 rats (control group, n = 20). The rats in group 2, on the other hand, received single focal intralesional injection of fresh PF4 (dosed at 20 μg/100 μL) for each lesion on day 0 and 2 of the intervention period as well. The tumour sizes were monitored daily for 5 consecutive days (i.e. from day 3 until day 8 of the intervention period) after the last PF4 or normal saline injection had been administered. No adverse events (e.g. reduced body weight, bleeding in the eyes, nose and ears, or death) were documented during the intervention period. All rats were subsequently euthanised via nasal carbon dioxide (CO_2) exposure at a rate of 2L/minute in a closed chamber. A summary of the experimental design and procedures is represented in Fig. 1.

Tumour sample collection

Breast tumour specimens were collected after there were changes in tumour sizes for 5 consecutive days following the allotted interventions. At necropsy, the rats were skinned off and the dissected skins containing intact tumours were photographed to record tumour location and size. The specimens were then carefully excised, fixed in 10% formalin for Haematoxylin and Eosin (H&E) and immunohistochemistry (IHC) stainings. The breast tumours were classified as invasive ductal carcinoma not otherwise specified (IDC-NOS), papillary, cribriform or ductal carcinoma *in situ* (DCIS) and these were further grouped into aggressive (IDC-NOS and papillary) and less aggressive (cribriform and DCIS) subtypes based on Jaafar and colleagues classification.[18]

Immunohistochemical analysis

The IHC staining was performed separately to demonstrate the expression of monoclonal caspase-3 (Abcam®, Cambridge, UK), polyclonal caspase-6 (Abcam®, Cambridge, UK), polyclonal caspase-7 (Abcam®, Cambridge, UK), polyclonal caspase-8 (Abcam®, Cambridge, UK) and polyclonal caspase-9 (Abcam®, Cambridge, UK). A standard-labelled streptavidin biotin (Dako®, Glostrup, Denmark) method was used on formalin-fixed paraffin-embedded tissue sections. The tissue blocks were trimmed and sectioned with a microtome (Leica®, Wetzlar, Germany) to obtain 3–5 µm thick sections

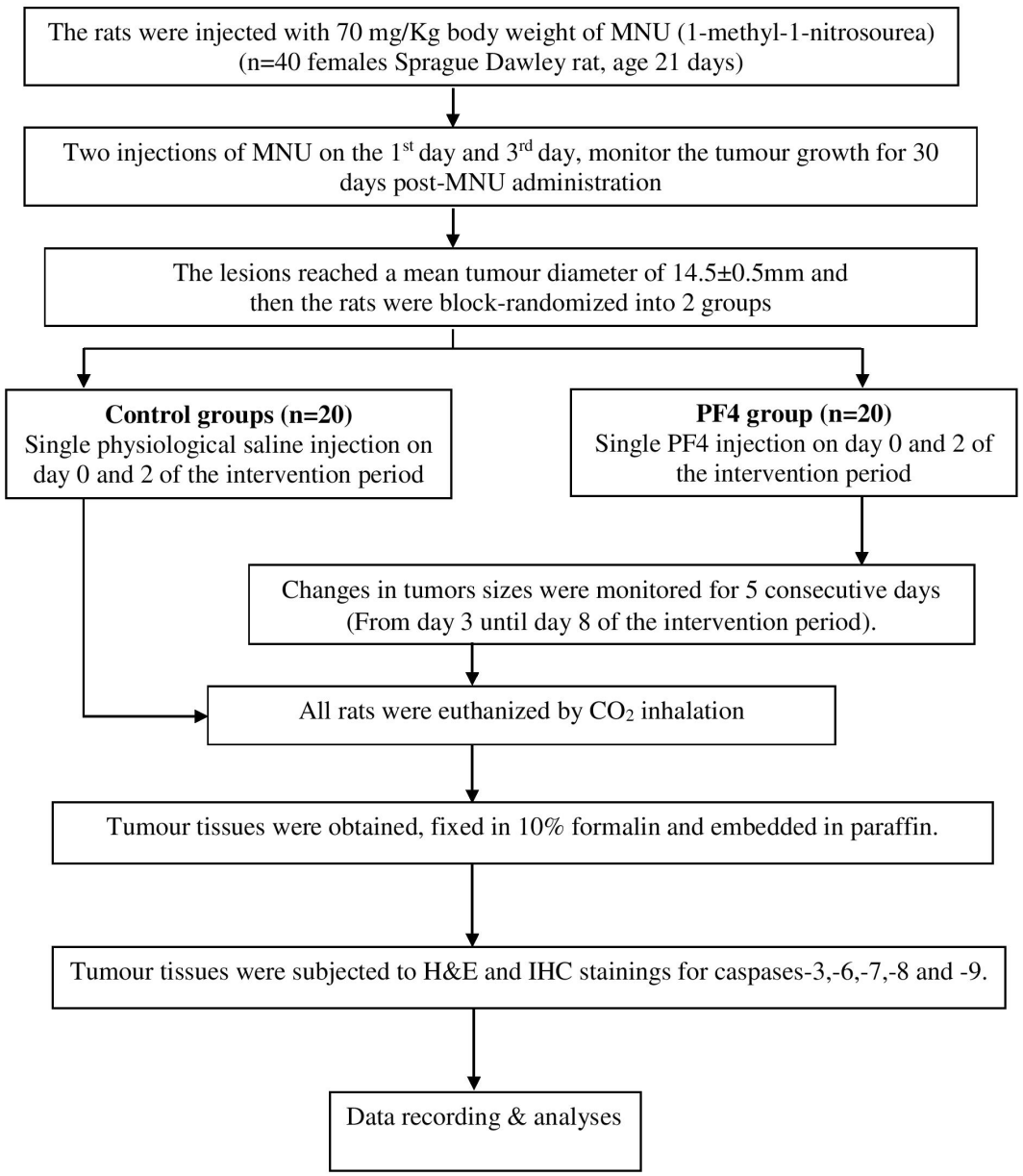

FIG. 1: The flow diagram of the experimental design and procedures.

of tissues which were later deparaffinised in xylene and dehydrated. Slides were then pretreated with Tris EDTA buffer (10 mM, pH 9.0, Sigma-Aldrich®, St Louis, Missouri) for 14 min and subsequently heated in a pressure cooker. Then, all the sections were treated for 10 min with peroxidase blocking reagent (DAKO® Glostrup, Denmark) to quench the endogenous peroxidase activity prior to incubation with primary antibodies. This was then followed by rinsing the slides with Tris buffered saline (pH 7.2, Sigma-Aldrich®, St Louis, Missouri).

The sections were incubated for 30 min with optimally diluted biotinylated secondary antibody and for 30 min with horseradish peroxidase afterwards, before they were ready for use. For visualisation, the slides were immersed in diaminobenzidine (DAB) (DAKO®, Glostrup, Denmark) substrate for 5 min, followed by washing in distilled water. The slides were then counterstained with Harris haematoxylin, dehydrated and mounted. To assess the reaction specificity, tissues from tonsillar region, breast carcinoma (CA), human skeletal muscle, normal

TABLE 1: The summary of procedural details for immunohistochemical analysis

Antibody	Clonality/positive control	Dilution	Optimisation
Anti-Caspase 3	Monoclonal/Tonsil	1:25 - 1:50	1:200/overnight
Anti-Caspase 6	Polyclonal/Breast CA	1/50 - 1/100	1:100/overnight
Anti-Caspase 7	Polyclonal/Human skeletal muscle	2 μg/mL	1:100/overnight
Anti-Caspase 8	Polyclonal/Colon	1:100	1:25/overnight
Anti-Caspase 9	Polyclonal/Colon	1:50 - 1:100	1:25/overnight

colon and colon CA were used as positive controls for caspase-3, caspase-6, caspase-7, caspase-8 and caspase-9, respectively. Negative controls (incubation without the primary antibody) were also used for this purpose (to test the specificity of an antibody). The procedural details are summarised in Table 1.

Immunohistochemistry Scoring System (ISS)

The light microscope (Nikon®, Tokyo, Japan) was used to examine the breast cancer tissues stained with IHC. The expression and immunostaining of caspases family were scored based on a previous study's recommendation.[23] Caspases family immunoreactivities were evaluated semi-quantitatively, based on the percentage of cells showing distinct diffuse cytoplasmic immunohistochemical reaction. Cytoplasmic immunoreactivities were assessed in at least five high-power fields (HPF) at ×400 magnification during which 100 cells were counted. If the tumour cells showed heterogeneous staining, the dominant pattern was utilised for scoring. The number of caspase-positive cells (brown) was recorded and its percentage (%) was calculated using the method mentioned previously.[22] The procedure was conducted thrice to obtain an average number of the cells.

Statistical analysis

Data was descriptively presented in mean (SD) / median (IQR) and frequency (%). The assumptions of normality and variance homogeneity were checked via Shapiro-Wilks and Levene's test, respectively. The differences in each caspase expression between the two groups were assessed either by the independent t or Mann-Whitney test. The Welch's version of the independent t test was utilised if variances were heterogeneous. All statistical analyses were carried out using Statistical Product and Service Solutions (SPSS) version 20.[24]

RESULTS

Morphological features of MNU-induced breast tumours

In total, 40 breast tumour specimens were collected. The MNU-induced breast tumours were mostly located at the mammary-thoracic region with nodular morphology (Fig. 2A). Tumour identification was facilitated by firstly identifying the nipples as a means to identify the mammary pad (Fig. 2B). The tumour specimens were then measured using Vernier caliper (Figs. 2C-D).

There is a significant reduction in the mean tumour size when comparison was made between the tumour size on day 0 and day 5 post-PF4 administration (14.5 mm (SD: 0.5 mm) vs 7.5 mm (SD: 0.5 mm), $p < 0.01$). On the contrary, the mean tumour size significantly increased in the placebo (physiological saline)-treated control group (day-0 mean tumor size: 14 mm (SD 0.5m) vs day-5 tumour size: 21 mm (0.5 mm), $p < 0.01$).

Types of MNU-induced breast tumours in intervention groups

Both control and PF4 groups exhibited similar number of aggressive and less aggressive tumour subtypes (Table 2). No significant associations were found between malignant tumour types and intervention types (Fisher's exact test, $p = 0.891$). The histological features of each tumour type had been morphologically described in Damitri and co-workers.[12]

Expressions of caspase family members

There were higher mean number of tumour cells expressing caspase-3 after treatment with PF4 as compared to control ($p < 0.001$). With respect to apoptosis executioners, there was significant increase in caspase-6 expression in PF4 group as compared to control ($p < 0.001$). However, the expression of caspase-8 was decreased in PF4 as compared to control ($p < 0.001$) (Table 3). The expressions of caspase-7 and caspase-9 were not significantly different between the

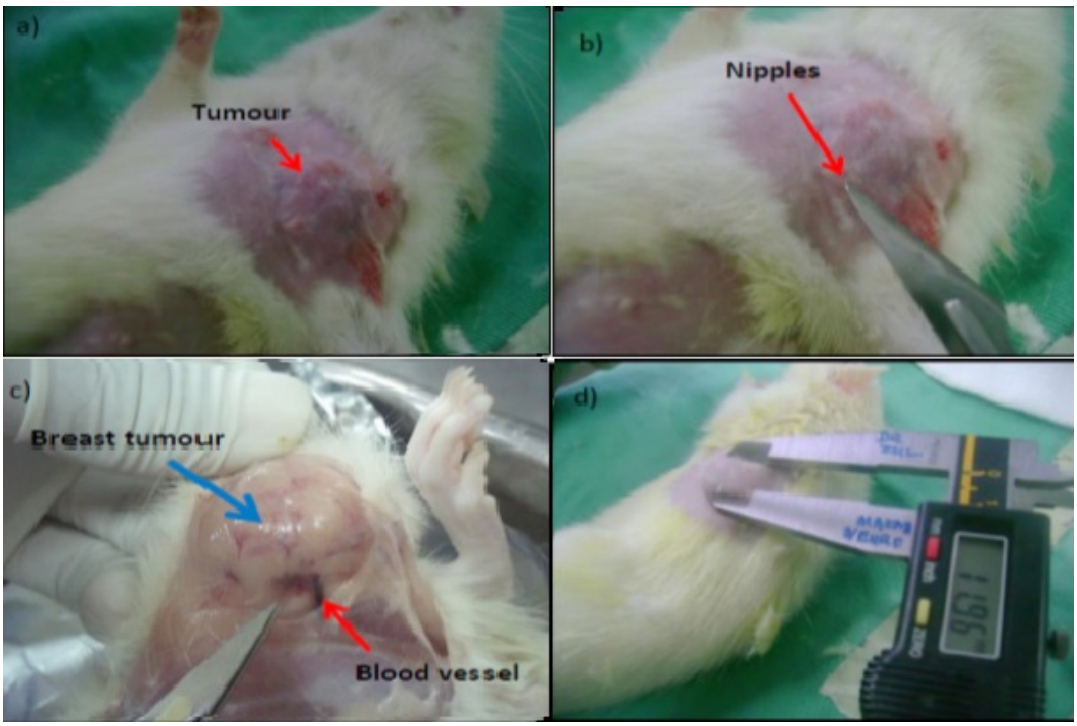

FIG. 2: The MNU induced tumour in SDR with a palpable lesion. (A) The tumour located at the cervical-thoracic mammary region. (B) The arrow showed the nipples of the breast as a means of breast pad identification. (C) the clearly-delineated vascularised mammary tumour (blue arrow) with an adjacent big blood vessel (red arrow). (D) The size of tumour was measured using digital caliper.

PF4 and control groups (p = 0.034, p = 0.061, respectively). Figures 3 (a-f) also indicated more pronounced caspase-3, -6 and -8 expressions in PF4 than the control group. However, the caspase-7 and -9 activities were similar in both groups (Fig. 3 g-j).

DISCUSSION

PF4, a constituent of C-X-C chemotactic cytokine class, can stifle tumour growth and metastasis by suppressing tumour-induced angiogenesis and induction of apoptosis machinery in many solid and non-solid tumours.[10,25] Besides, PF4, delivered into the target cells via retroviral vector, was found to downregulate Vascular Endothelial Growth Factor (VEGF) expression in murine models and various human tumour cell lines.[26] In this study, we assessed the PF4 effects on the expression levels of caspase-3,-6 and -7 (executioner caspases) and caspase-8 and -9 (initiator caspases), the major players of intrinsic and extrinsic apoptosis pathways.

TABLE 2: The distribution of malignant tumour stratified by the degree of aggressiveness in both PF4 and control groups (n = 40)

	Control (n=20)	PF4 (n=20)	Total (n)
Aggressive			
IDC-NOS	8	8	16
Papillary	5	4	11
Total (%) of tumor	65	60	
Less Aggressive			
Cribriform	3	5	17
DCIS	4	3	11
Total (%) of tumor	35	40	

TABLE 3: The differences in caspase-3, -6, -7, -8 and -9 expression between the groups based on IHC

Caspase	Mean (SD)		Mean difference (95% CI)	t-stats (df)	p values
	PF4 (n = 20)	Control (n = 20)			
Caspase-3	82.8 (4.34)	54.6 (14.11)	21.4 (14.4, 28.4)	8.546 (22.56)[a]	**< 0.001**
Caspase-6	84.5 (6.57)	62.7 (18.30)	21.8 (12.88, 30.82)	5.027 (23.81)[a]	**< 0.001**
Caspase-7	93.0 (15.75)[b]	91.0 (16)[b]	2[c]	165.50[d]	0.347
Caspase-8	63.5 (15.1)	91.1 (7.86)	27.6 (19.80, 35.40)	7.239 (28.55)[a]	**< 0.001**
Caspase-9	89.4 (8.28)	86.8 (9.87)	2.6 (-3.23, 8.43)	0.902 (38)	0.373

[a]Welch's independent t-test (corrected df for unequal variances); [b]Median (IQR); [c]Median difference; [d]Mann-Whitney U test statistic

We demonstrated the effects of PF4 on the expression level of each caspase involved in apoptosis execution. Caspase-3 expression was significantly increased after tumour cells were treated with PF4 and this is translated into reduction in tumour growth and improved survival of SDRs with MNU-induced breast tumours. We may conclude that PF4 exerts its anti-tumour properties by upregulating caspase-3 expression level. This is further corroborated by the fact that caspase-3 is the principal caspase for catalysing the cleavage of many key cellular proteins within the apoptosis machinery.[27]

We also observed that caspase-6 expression level was also significantly raised in PF4-treated group. Our finding is in tandem with Vakkala and colleagues who demonstrated a significant association between increased caspase-6 expression level and raised apoptotic index and breast lesions' aggressiveness.[28] Furthermore, our claim is further substantiated by Roth and co-workers who revealed that caspase-6 and -8 are critical for apoptotic execution in both dysplastic breast tissues and breast carcinoma.[29]

The similar executor caspase-7's expression level in both PF4 and control groups can be explained by the redundant and overlapping functions of both caspase-3 and -7. Walsh and colleagues demonstrated that caspase-3 is the primary effector caspase which is adequate enough to cleave the majority of substrates (with the exception of cochaperone p23) at the end stage of apoptosis.[30] Apart from that, Slee and coworkers also showed that only the removal caspase-3 (not caspase-7) eliminated the proteolysis of most caspase substrates induced by cytochrome c/dATP in human Jurkat cell-free extracts.[31] This is further corroborated by Bretnall and co-workers who confirmed that caspase-7-deficient mouse embryonic fibroblast (MEF) cells were still sensitive to intrinsic apoptosis.[32] These findings are in line with our finding that showed caspase-7 has little role in PF4-mediated breast cancer cell apoptosis.

Apart from that, caspase-8 expression level was also significantly higher in PF-4 treated group than the control group. On the other hand, the expression level of caspase-9 was not significantly different in both PF4 and control groups. We postulate that the finding implies that PF4 induces breast cancer cell apoptosis via the intrinsic apoptosis pathway and not the extrinsic apoptosis pathway. This is corroborated by a prior study which demonstrated that Homeobox A5 (HOXA5)-induced breast tumour cell apoptosis was mediated by caspase-8.[33] Besides, a recent study also showed that caspase-8 expression was reduced in breast tumour specimens compared to the adjacent normal breast tissues in a group of Iranian breast cancer patients.[34] We believe these supports our hypothesis that PF4 may exert its apoptotic effects by increasing caspase-8 expression. Based on our previous findings[12] and current findings, a proposed schematic representation of PF4 effects on the molecular pathway of apoptosis is shown in Fig. 4.

There are several limitations in this study. Firstly, we did not investigate the precise location where PF4 exerts its effects on the extrinsic apoptosis pathway. We hypothesised that the PF4 modulates the extrinsic pathway of apoptosis upstream of caspase-8 such as at the DISC or FADD recruitment levels. Besides, we didn't elucidate the precise nature of mechanism of PF4 effect on the caspase-independent pathway. Apart from that, there is a possibility that the PF4 may

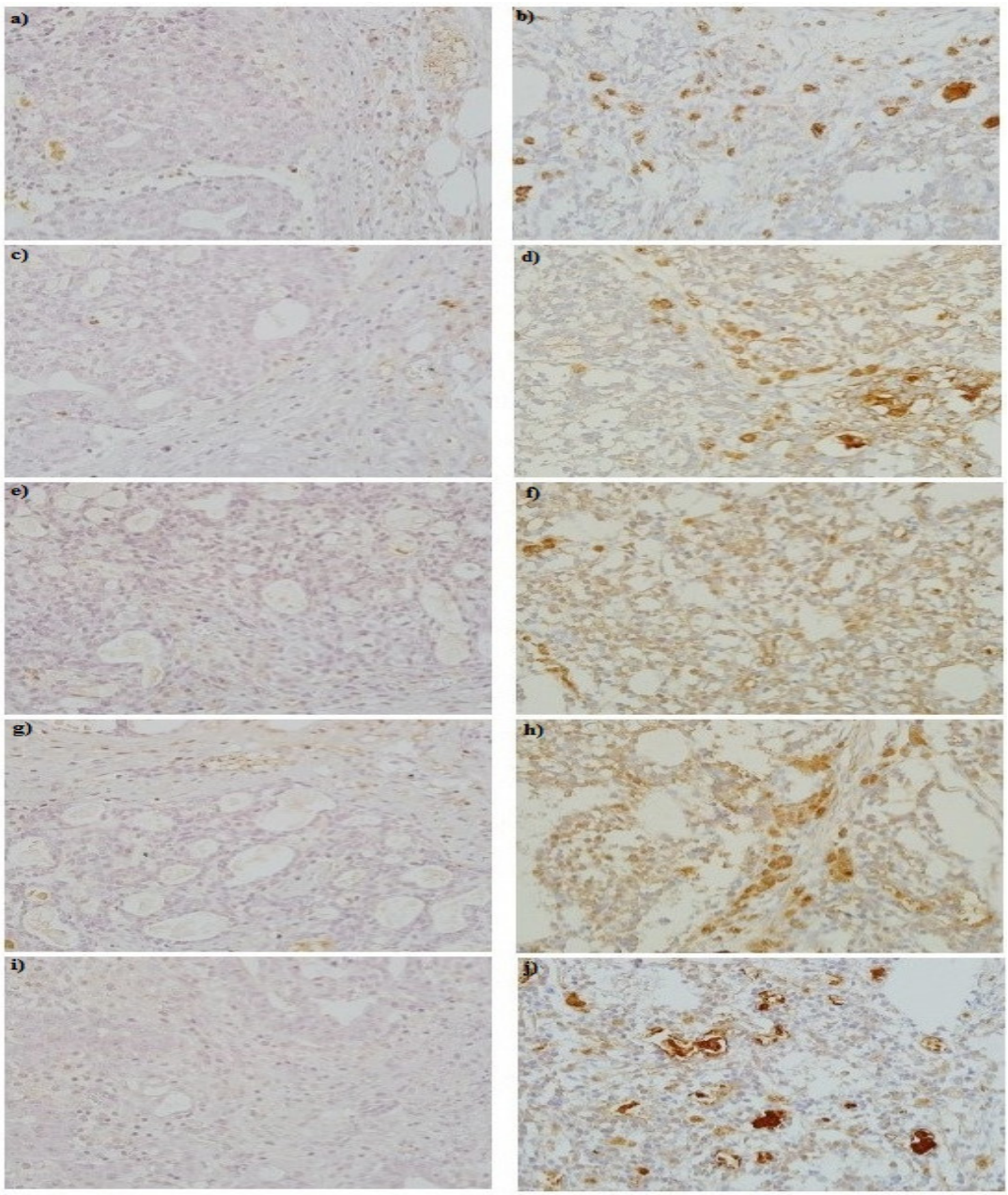

FIG. 3(A-J): Histological sections showed more positive caspase expression of caspase-3, caspase-6 and caspase-8 (brown colour) in PF4-treated breast tumour specimens (B, D and H) than the control group (A, C and G) whilst caspase-7 and caspase-9 expressions were similar in both PF4 (F and J) and control (E and I) groups (IHC, x400).

have influence on the other aspect of intrinsic apoptosis pathway modulated by the second mitochondria-derived activator of caspase (Smac / DIABLO) and Omi / HtrA2 pathways which may increase the blocking effects on inhibitors of apoptosis proteins (IAP) such as X-linked Inhibitor of Apoptosis Protein (XIAP), cellular Inhibitor of Apoptosis Protein-1 and -2 (cIAP 1 and cIAP2) which in turn remove their direct block on the caspase-3 and -7 activation. These will be addressed by the ongoing research that we are currently conducting.

In conclusion, we proposed that the apoptosis effect of PF4 is modulated through the intrinsic apoptosis pathway through caspase-3, -6 and -8 activation. We have found no significant PF4

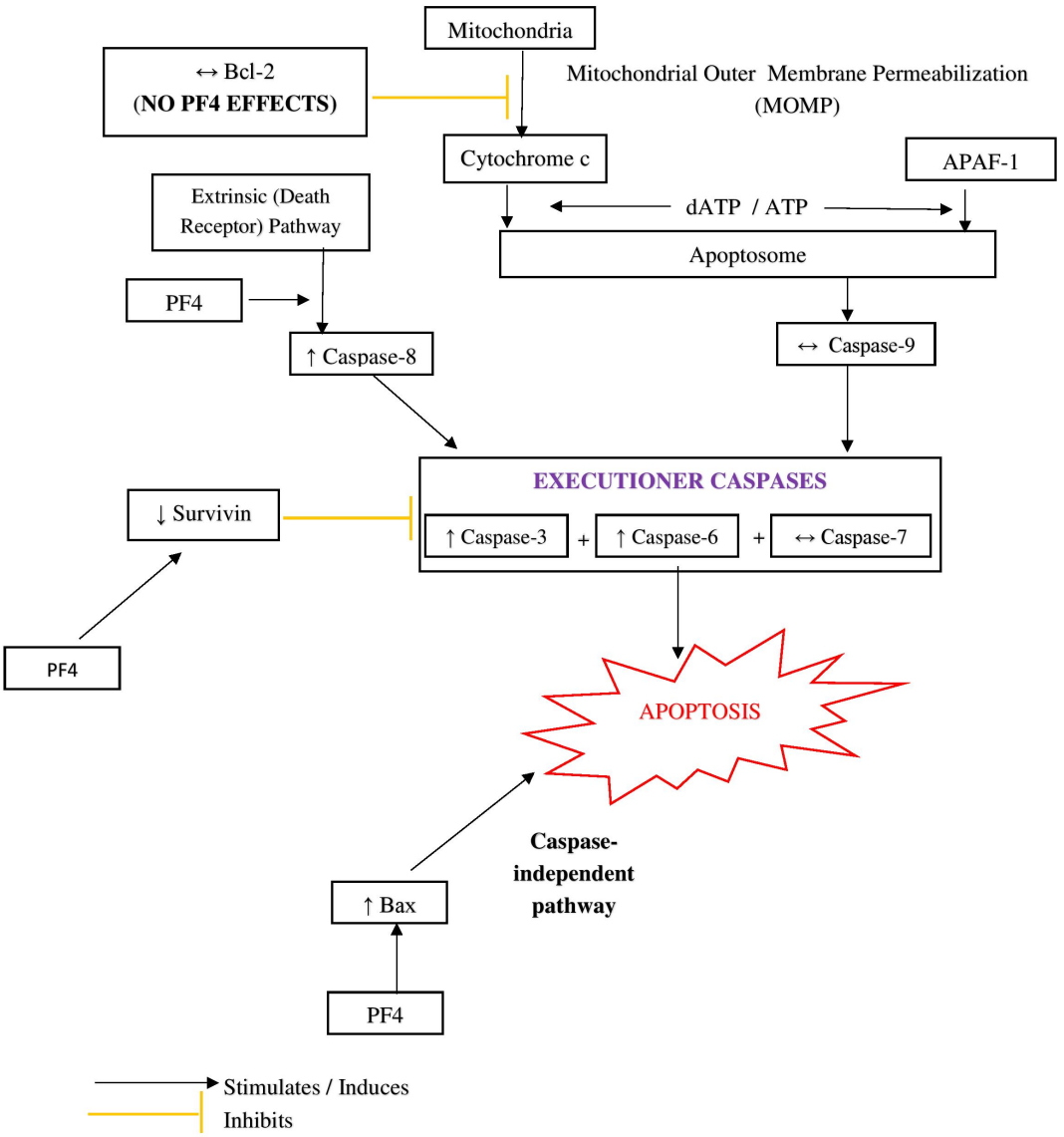

FIG 4: A proposed schematic representation of PF4 effect on the caspase-mediated pathway of apoptosis.

effect on caspase-9 expression and thus negating its direct effect on the intrinsic pathway via head-on caspase-9 activation. However, further study is required to elucidate the PF4 effects on other upstream constituents of the intrinsic and extrinsic pathways of apoptosis.

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Conflict of Interest: The authors declared no conflict of interest.

Author contributions: Conceived and designed the experiments: TAD, MIAJ, AS, SS, HJ. Performed the experiments and data collection: TAD. Analysed the data: MIAJ, TAD. Contributed reagents/materials/analytic tools: SS, HJ. Planning, drafting and finalising the manuscript: MIAJ, TAD.

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